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## HPLC approach for revealing age-related changes of aquatic dissolved organic matter in sediment core

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### Abstract

Analytical method based on HPLC has been used to characterize aquatic dissolved organic matter (DOM) from sediment core of Lake Võrtsjärv, South- Estonia. High-performance size exclusion chromatography (HPSEC) as separation method was coupled with diode-array detection (DAD) and separated molecular fractions of DOM were subject to qualitative and semi-quantitative analysis. Qualitative analysis based on UV- spectra revealed the presence of proteins and humic constituents in separated high molecular weight fraction and aromatic constituents in low molecular weight fraction. Statistical data treatment methods enabled clustering sediment layers into 4 periods according to sediment depth and age. The upper 0-30 cm sediment DOM had statistically relevant differences in comparison to other periods as revealed by lower total peak and humic substances (HS) fraction areas, and molecular weights. Samples from 80-120 cm depth differed from others by decreased low molecular weight (LMW) fraction content. The observed down-core trends suggest polymerization of LMW organic constituents and increasing humification. The statistical analyses revealed that some chromatographic and spectrometric parameters can be used to differentiate between sediment layers and to evaluate environmental changes.

**Keywords:** HPLC; HPSEC; dissolved organic matter; humic substances; sediment

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### 1. Introduction

Sedimentary organic matter (OM) plays an important role in many geochemical and biochemical processes in aquatic environments. Sedimentary OM is an extremely complex and heterogeneous mixture of humic substances (HS), proteins, lipids, carbohydrates, and other biomolecules. OM is formed during the degradation process of higher plant detritus and the composition of microbial cellular material [1-3]. It is distributed in aquatic environments as particulate and dissolved compounds. The investigation of aquatic OM gives an overview of the processes going on in the studied environment (polymerization, degradation, etc). The types and amounts of OM in lake sediments present a paleolimnological record of past conditions in the lake and its catchments area. Thus, there

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is interest in investigation and characterization of sedimentary OM from lakes as it allows predicting and evaluating temporal changes in the studied ecosystem [4].

Humic substances compose the main part of dissolved organic matter (DOM). HS are formed by the association of components during the humification process. The source of aquatic HS is thought to be the formation from phytoplankton in the water or they might be washed into waters from terrestrial and soil deposits.

Since DOM is naturally a very complex mixture of molecules, the determination of its exact chemical composition is a complicated task. Only detailed chemical characterization using various analytical methods could be carried out. A part of DOM is optically active, enabling usage of spectroscopic methods based on UV absorption for the characterization. Another possibility of DOM analysis is the measurement of molecular size and molecular weight distribution by using HPLC with size exclusion option (high-performance size exclusion chromatography HPSEC) [5-7]. When HPSEC as separation method is coupled with diode-array detection (DAD) it is possible to obtain and store spectra of molecular fractions of DOM for its subsequent qualitative and semi-quantitative analysis. Great advantage of HPSEC-DAD approach is the non-destructive analysis, small sample volume and minimal sample pretreatment, thus making method suitable for environmental studies.

The aim of present study was to separate DOM constituents from 2000-year old sediment core and to test HPLC in respect to revealing the age- related changes.

## 2. Experimental

### 2.1. Study site and sampling

The Lake Võrtsjärv sediment samples were taken in 2003 from the ice-cover. The sampling site situated in the southern part of the lake where water depth was 1.40 m (Table 1). Different coring devices were used for acquiring unmixed, continuous sediment records without disturbances (more details on the study site and sampling can be found at [8]).

**Table 1.** Characteristics of Lake Võrtsjärv, South-Estonia

Location	58°17'N 26°02'E
Coring site	58°09'42"N 26°04'10"E
Origin	glacial
Area, km <sup>2</sup>	270
Average depth, m	2.8
Maximum depth	6
Catchments area, km <sup>2</sup>	3374
Trophic status	eutrophic

The frozen sediment core (120-cm long) was sliced into 1-cm thick sub-samples and packed into plastic bags. Pore water was extracted from thawed sediments by centrifugation at 3500 rpm for 30 minutes, filtered through 0.45 µm filters (Millex, Millipore) and stored at 4 °C in the dark.

The chronology of Lake Võrtsjärv sediments was established and evaluated by different independent approaches as <sup>210</sup>Pb dating; <sup>137</sup>Cs and <sup>241</sup>Am reference horizons and indirect dating by fly-ash particle distribution in the sediments [9-10].

## 2.2. HPLC analyses

The molecular characteristics of DOM were determined using a HPLC system which comprised of a Dionex P680 HPLC Pump, Agilent 1200 Series (Agilent Technologies, UK) diode array absorbance detector (DAD), a Rheodyne injector valve with a 50  $\mu$ L sample loop. A BioSep-SEC-S 2000 PEEK size exclusion analytical column (length 300 mm, diameter 7.50 mm, Phenomenex, USA) preceded by a suitable guard column (length 75, diameter 7.50mm, Phenomenex, USA) was used for separation. The applied flow rate was 1.0 mL min<sup>-1</sup>. The column packing material was silica bonded with hydrophilic diol coating, with particle size of 5 $\mu$  and pore size of 145Å. The mobile phase consisted of 0.10 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> - (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> buffer with pH 6.8. The HPLC system was calibrated using 5 different molecular weight protein standards (Aqueous SEC 1 Std, Phenomenex, USA). All solutions for HPLC measurements were prepared using distilled water passed through MilliQ water system, filtered with 0.45  $\mu$ m pore size filters (Millipore) and degassed. Samples were analysed in triplicate. For quality control the aqueous protein standard was analysed each day. The chromatograms were recorded and processed by Agilent ChemStation software. Full details of the used method are described previously [7].

It has been found that charge repulsion effects of the size exclusion packing material and adsorption interactions with the humic compounds can affect the measurement results with HPLC [6, 11]. Only UV-absorbing compounds have been detected by used method. Aliphatic fraction of DOM was not measured because of the absence of conjugated double bonds.

Total chromatogram peak areas, representing the total UV-absorbing fraction of the DOM in each pore-water sample were used in the data analysis. The peak area corresponds to the quantity of DOM in a specific molecular size fraction. Respectively the peak with the lowest retention time refers to the highest molecular size fraction of DOM and peak with the highest retention time to the lowest molecular size fraction. Peak areas as semi-quantitative characteristics were used in presenting age-related variations of DOM fractions. To obtain the percentages of molecular fractions, the chromatograms were divided into three molecular size fractions: below 150 Da (low molecular weight, LMW); 1.2 - 2.3 kDa (humic substances, HS) and 530–1,200 kDa (high molecular weight, HMW) and the areas of the respective fractions were divided by the total peak area. Weight-average and number-average molecular weights of DOM ( $M_w$  and  $M_n$ , respectively) were determined using the formulas:  $M_w = \Sigma(h_i M_i) / \Sigma h_i$  and  $M_n = \Sigma h_i / \Sigma (h_i / M_i)$ , where  $h_i$  was the detector output and  $M_i$  was the molecular weight, both at the  $i$  th retention time [12]. The polydispersity  $M_w/M_n$ , describing the homogeneity or heterogeneity of organic matter was calculated from obtained data.

## 2.3. Spectroscopic analyses

Absorbance spectra of the samples were collected with the Jasco V-530 UV/VIS Spectrophotometer (Japan), with 1-cm-pathlength fused silica cells and MilliQ water as the blank. Spectra were measured over the range of 200 - 500 nm with 2.0 nm bandwidth. The absorbances ratio at wavelengths 250 to 360 (A<sub>250/360</sub>) was calculated from spectra. Absorption coefficients were calculated using formula  $a_{CDOM}(\lambda) = 2.303 A(\lambda)/L$ , where  $A(\lambda)$  was the absorbance at selected wavelength and  $L$  the pathlength of the cell in meters.

## 2.4. Statistical analyses

Cluster analysis using the Ward method was applied to reveal age- related periods in analyzed samples [13]. The analysis was performed on the chromatographic and spectroscopic data. As descriptors, all of the separated peak areas and total chromatogram areas, molecular weights and their ratios, DOC and A<sub>250/360</sub> for all samples were included into the analysis. The Euclidean distance as a measure of similarity-dissimilarity of samples was used. Factor analysis was conducted on data as well using principal components method [14-15]. Both statistical analyses were carried out with WinSTAT for Excel software (R. Fitch Software, Germany).

### 3. Results and Discussion

#### 3.1. Characteristics of HPLC separated DOM fractions

Usually 3-7 different peaks were separated in analysed L. Võrtsjärv sediment pore water samples. An example of HPSEC chromatogram is provided in Figure 1. The HPSEC profiles were dominated by HS fraction in all analysed samples except surface layers samples where LMW fractions dominated (Figure 2). The intensities and positions of the peaks (*i.e.* fractions) changed in different sediment layers reflecting the age-related changes in the concentration and transformation of organic constituents.

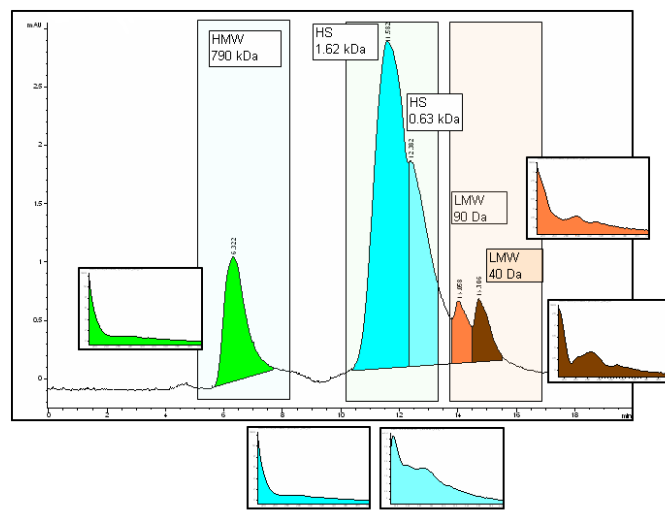


Fig. 1. HPSEC chromatogram of Lake Võrtsjärv pore water sample from sediment layer at 111-112 cm depth, UV detection at 280 nm. The DOM molecular fractions are eluted in order of decreasing molecular sizes; molecular weights are shown on top of each peak; integrated UV-spectra for separated peaks are shown in separated windows differentiated by similar colours.

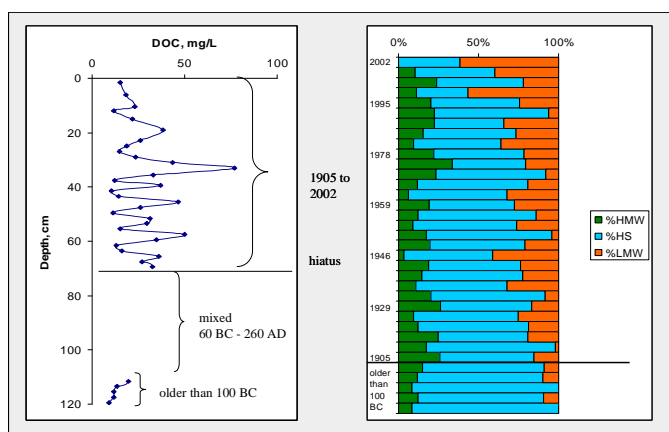


Fig. 2. Age-related changes in DOC content and separated molecular fractions of L. Võrtsjärv pore waters (HMW- high molecular weight, HS- humic substances, LMW- low molecular weight).

The qualitative analysis of DOM components was based on peaks UV- spectra obtained with DAD detection. The HMW fraction had characteristic humic-like but also protein-like UV- spectra suggesting the occurrence of HS colloids and in some sediment layers proteins. The proteins and organic colloids have been found to be present in DOM samples [16-17]. The HMW ( 530 – 1200 kDa) fraction composition, in respect to proteins, was studied in more detail by fluorescence spectroscopy in the previous study [18]. In samples, dated older than 100 BC the protein-like spectra dominated. The presence of proteins in HMW fractions could be explained with the association of proteins and humic compounds which obviously made them not available for bacterial degradation and thus detectable in older samples. The HS fraction was characterized by a broad peak with characteristic molecular weights of aquatic humic and fulvic acids (1200 – 2300 Da), with shoulder (600 – 800 Da), and usually differing UV- spectra of those peaks (Fig.1). The compounds which eluted as subpeak had more aromatic character. LMW fraction (< 130 Da) was separated up to 5 peaks in samples from 20th century. UV- spectra had characteristic maxima of aromatic structures at wavelengths 225 to 270 nm. In the oldest samples (< 100 BC), the number of LMW peaks decreased to 2 and samples had even more aromatic structures as revealed by UV- spectra (maxima at wavelengths 270 – 290 nm). The aromatic structures absorbing at 280 nm have been associated with the structures of lignin origins [19]. The exact identification of separated LMW aromatic compounds was not possible by used HPSEC-DAD method. Usually LMW components present in aquatic samples are amino acids, amino sugars, nucleic acids and carboxylic acids. The organic acids could be derived from the oxidation of lignin structures or from microbial or photochemical degradation of cellular macromolecules [20].

### 3.2. Age-related changes of DOM characteristics in analyzed sediment core

The statistical analysis of data was performed to reveal periods in separated DOM fractions characteristics. Cluster analysis identifies the homogeneous subgroups, within dataset. Based on cluster analysis including all variables, the data were operationally grouped into 4 depth/age-periods: I) 0 – 30 cm of sediment core depth, dated to 2002 – 1997 and named *recent*; II) 30 – 52 cm core depth, dated to 1972 – 1943, and named *eutrophication*; III) 53 – 80 cm core depth, dated before year 1940, and named *before eutrophication*; IV) 81- 120 cm core depth, dated before 100 BC and named *background*. However, HMW, HS and LMW peak areas allowed data clustering only into 2 periods. The mean values of analyzed variables divided into 4 periods (I to IV) with 95% confidence limits are shown in Figure 3. The absorption coefficients  $a_{\text{CDOM}}(254)$  were following similar down-core trend as DOC, with the mean value of 172, and the maximum value of 418 at sediment depth 30-32 cm. The DOC, total chromatogram peak area and HS peak area also changed similarly, thus proving the suitability of peak areas as semi-quantitative characteristics of DOM. The upper 0-30 cm sediment DOM had statistically relevant differences in comparison to other periods as revealed by total peak and HS fraction areas, and molecular weights. The recent DOM accumulating into sediments has lower molecular weights and lower HS contents in comparison to preceding sediment layers.

Samples from IV period (80-120 cm depth) differed from others by low LMW content. Observed down-core trend suggest polymerization of LMW organic constituents and increasing humification as revealed by the highest content of HS. It was not possible to statistically differentiate between periods II and III, using obtained chromatographic and spectroscopic DOM characteristics, although slight difference could be detected in HS content. To conclude about eutrophication of aquatic environment the addition of nutrient data might be essential.

The principal component analysis (PCA) was conducted for all the data obtained for Lake Võrtsjärv in present study to find the possible chromatographic and/or spectroscopic variables that could possibly indicate changes in the environment. The PCA plots are shown in Figure 4. In *recent* sediment layers the validated variance was 64.5% for the first two principal components (46.1% for PC1 and 18.4% for PC2). In *background* layers the first principal component explained 51.9% and the second 22.9% of the variation in the data. In comparison of periods I and IV (*recent* and *background*) we observed some variables changing their location. The HMW and LMW contents changed their positions on PCA plots. In *background* layers, HMW was grouped together with total peak and HS areas and DOC (similar second PC); in *recent* sediments, it was clearly differentiated by PC2. The change was detected for HS and LMW contents, absorbance ratio and DOC, meaning all those variables could be used to differentiate sediment layers. The PCA plots of the *eutrophication* and *before eutrophication* periods (II and III) were quite similar. The total peak- and HS areas positions were changed along PC1 axis. The polydispersity index

Mw/Mn slightly moved along PC2 axis. Thus total chromatographic peak - and HS areas could serve as possible indicator variables.

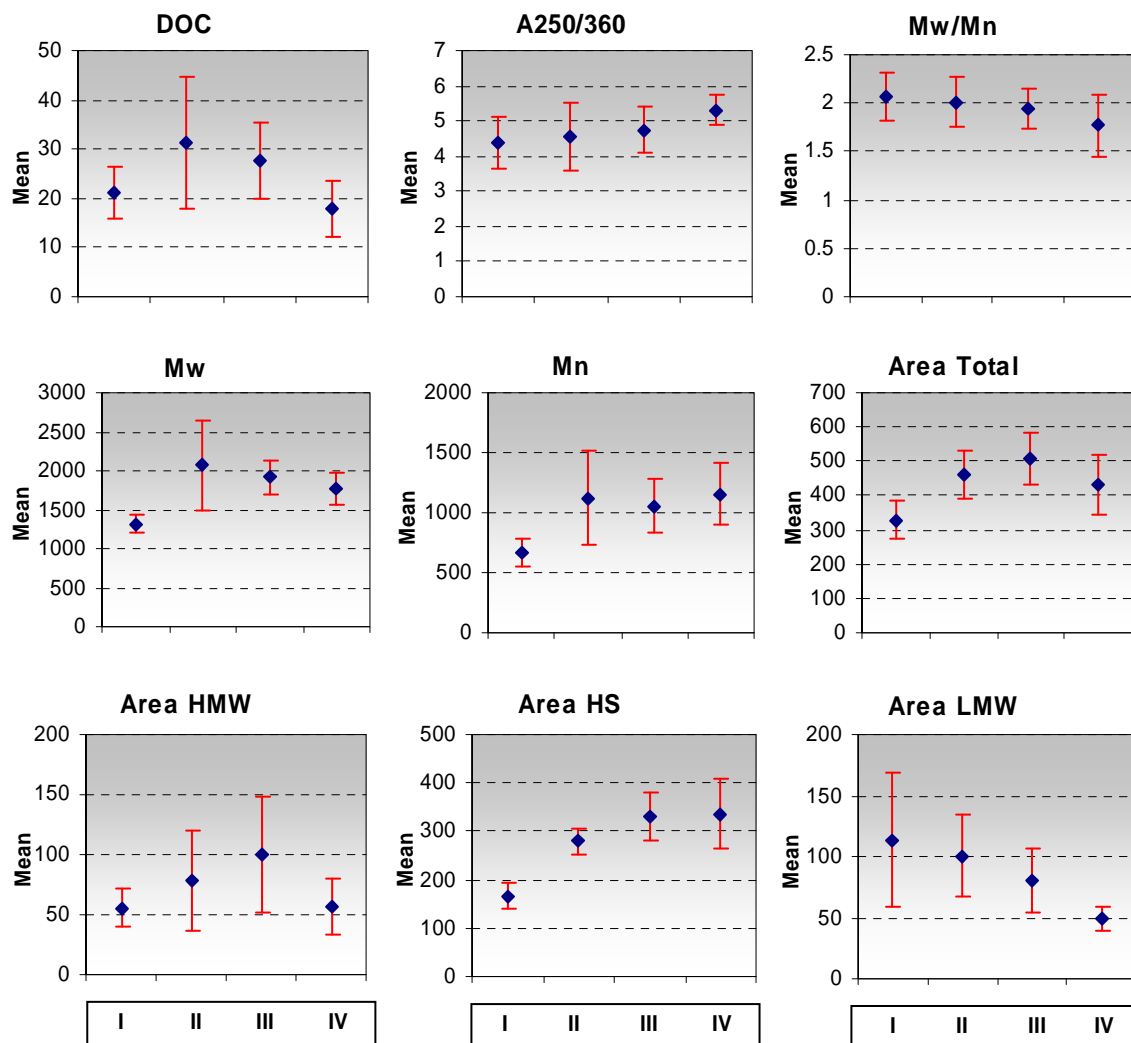


Fig. 3. Plots describing mean values of DOM semi-quantitative (areas) - , molecular - and spectroscopic characteristics arranged into 4 age/depth-periods (see text for description). Red bars indicate confidence limits at 95% level. DOC, mg/L; A250/360- absorbance ratio at respective wavelengths; Mw/Mn- polydispersity; Mw and Mn- weight- and number- average molecular weights, respectively, Da; Area Total- total chromatogram peak area, Area HMW, Area HS, Area LMW – HMW, HS, LMW fraction peak area, mAU\*s.

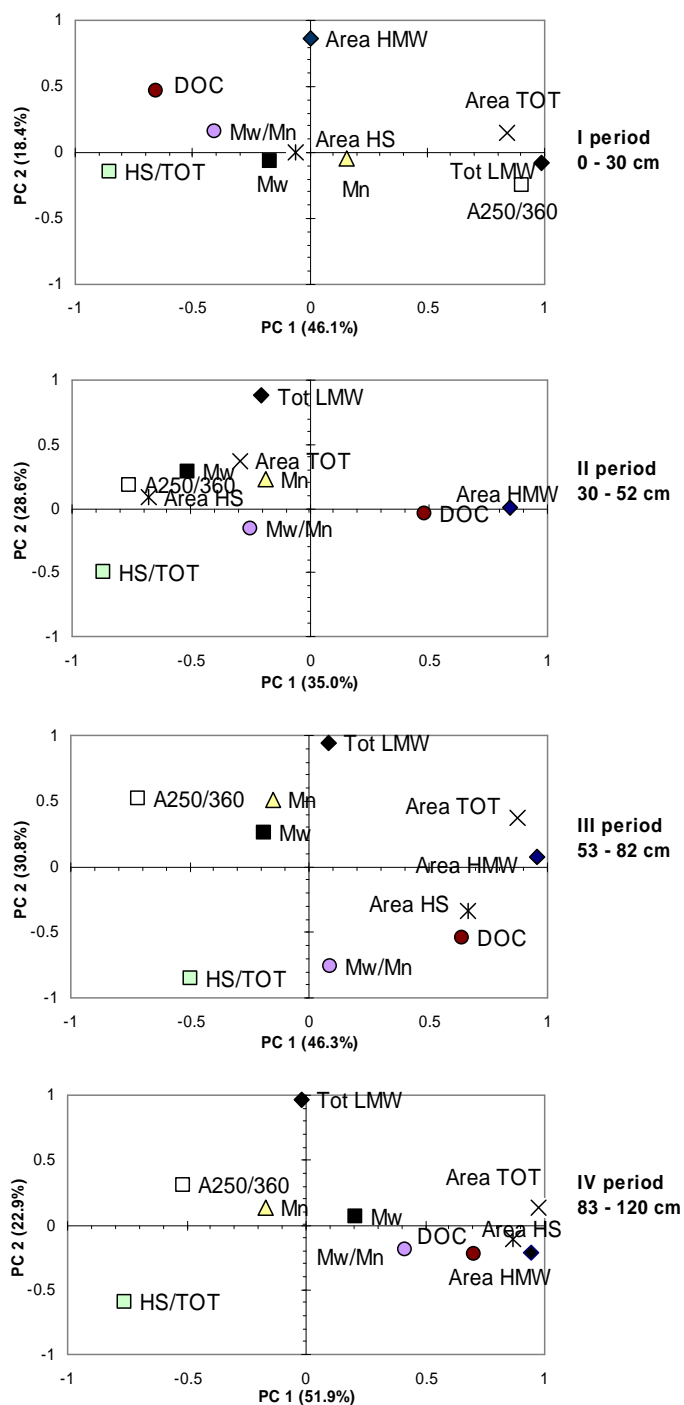


Fig. 4. Principal components showing Lake Vörtsjärv chromatographic and spectroscopic variables in 2000- year old sediment core differentiated by depth/age periods.

#### 4. Conclusion

The results of sediment core pore water analyses demonstrated that the HPSEC-DAD method is potentially a promising tool of investigating changes in sediment organic matter. The observed down-core trends suggested polymerization of LMW organic constituents and increasing humification in Lake Võrtsjärv sediment core. HS was dominating separated fraction which was increasing with the increasing sediment depth and age and had characteristic molecular weights of aquatic humic and fulvic acids. The statistical analyses revealed that some chromatographic and spectrometric parameters can be used to differentiate between sediment layers and to evaluate environmental changes. Additionally the DOM characteristics can provide insights to its sources.

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